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UPDATED REVIEW ON LC-NMR

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ABSTRACT

NMR is considered to be one of the least sensitive methods among the various spectroscopic techniques available to date. The first on-line HPLC-NMR coupled analysis was carried out using super conducting magnets. There are various NMR probes that can be used for increasing the efficiency of LC-NMR. This review explains the balancing of both LC-NMR for attaining the sensitivity and accuracy of both the techniques. However new upcoming challenges in the future can be solved by using this technique, due to the development of the new cryogenic LC-NMR probes which coupled with the recent interface enhancement and higher magnetic field strengths NMR has become one of the most powerful and versatile spectroscopic techniques for the analysis of bio-macromolecules, allowing characterization of bio-macromolecules and their complexes. Specifically, interfacing liquid chromatography with parallel NMR and mass spectrometry (LC–NMR–MS) gives comprehensive structural data on metabolites of novel drugs in development. Applications in natural product, combinatorial chemistry and drug metabolism studies are reviewed.

KEYWORDS: LC-NMR, NMR probes, Super conducting magnets, bio-macromolecules.

1. INTRODUCTION

Whenever the chromatographic techniques and the spectrometric methods are combined online are called hyphenated techniques[1], and they have attracted attention in recent years as high-throughput analytical methods that provide separation of mixtures at the same time as the spectra of the various components since, among this detailed structural information can be obtained from LC-NMR that combines high-performance liquid chromatographs (HPLC) and nuclear magnetic resonance spectrometers (NMR),(Fig . 1) they have been applied widely in

the analysis of complex mixtures that contain unknown components such as impurities and metabolites in pharmaceuticals, natural products and synthetic polymer[2,3], ever since they were 1st reported in 1978.[4]

On the other hand, looking at this from the standpoint of applications in research and development at manufactures, it can't be said that they have been sufficiently practical in terms of sensitivity and operability of equipment up to now, and they are not a wide-spread ttechnique compared with LC-MS.

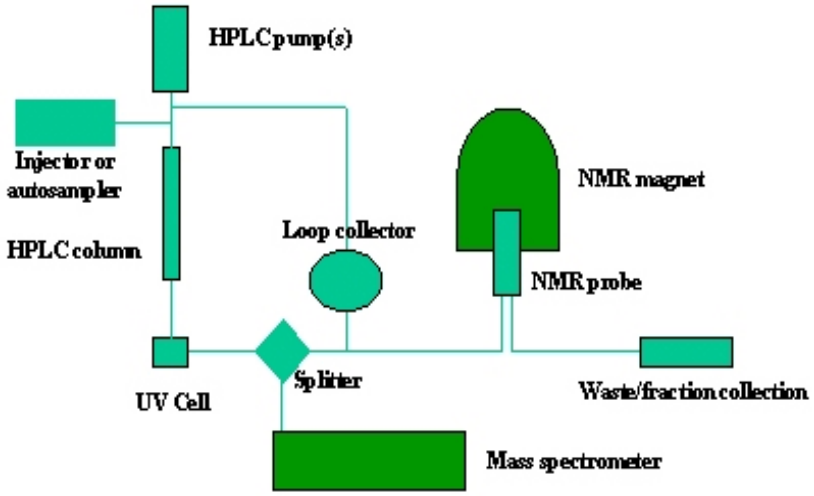

Fig. 1: Schematic repersentation of LC-NMR.

Since the 2000's, however there were important favourable changes in the situation, and currently, LC-NMR has become a highly practical analytical method because of increased sensitivity in the NMR devices, such as highly magnetic field magnets and highly sensitive probes, and maturation of peripheral technologies, such as solvent elimination technology and automatic measurement software suitable for multi-component analysis. e.g., Application for research and development in find chemicals, pharmaceuticals and agricultural chemicals. Further combination technologies that linked LC-NMR with other detectors have appeared in recent years, and become possible to acquire multiple spectra simultaneously.

2. History

In spite of the fact known that this approach is time consuming and technically demanding, both the LC and NMR have been and are still routinely used in the mixture analysis. In theory, the physical coupling of LC and NMR could save a lot of time and was proposed over 30 years ago. Even then the successful and applicable coupling of LC-NMR was achieved in

past two decade. The first on-line LC-NMR experiments were performed in late 1970's by Watanabe and Niki who demonstrated stopped-flow measurements of mixture of known compounds. The conventionally used NMR probes was converted to the flow through probe by the use of the thin-walled Teflon capillary with in a standard NMR tube and spectra were recorded with sample rotation.[5]

The 1st real sample to be analyzed by LC-NMR technique was a military jet fuel using the normal phase columns and deuterated chloroform and freon.[6] After the advance made the combination of LC-NMR was made. LC-NMR and LC-MS are considered to be the most valuable techniques for the structure elucidation of the unknown compound in wide field of application. This technique is essential for analysis of products obtained from natural sources because various closely related substances are present in their extracts, which are difficult to separate. It is important to note that substances derived from plant origin are almost containing 40% of newly registered compound present in the drug discovery program.

Thus, there is the need for development of new innovative technique that can describe the profile of each and every component of complex mixture and that to in a very simple way as well as fast procedure, this has become a challenge and this is to be looked forward into. Recently, there are various LC-NMR system available, and data acquisition can be accommodated with the help of different modes depending on the status of the sample during investigation.

3: Basic Principles of LC-NMR

3.1 Hplc

High performance liquid chromatography[7] (HPLC) is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressure of up to 400atmosphere.That makes it much faster. All chromatographic separation, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

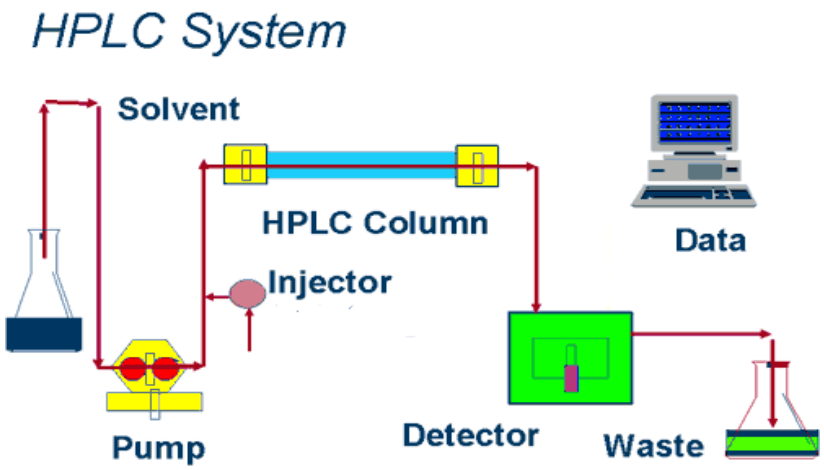

Figure. 4.1: Instrumentation of HPLC.

3.2: NMR

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the basic energy to a higher energy level (generally a single energy gap). The energy transfer take place at a wavelength that corresponds to radio frequencies and when spin returns its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned.

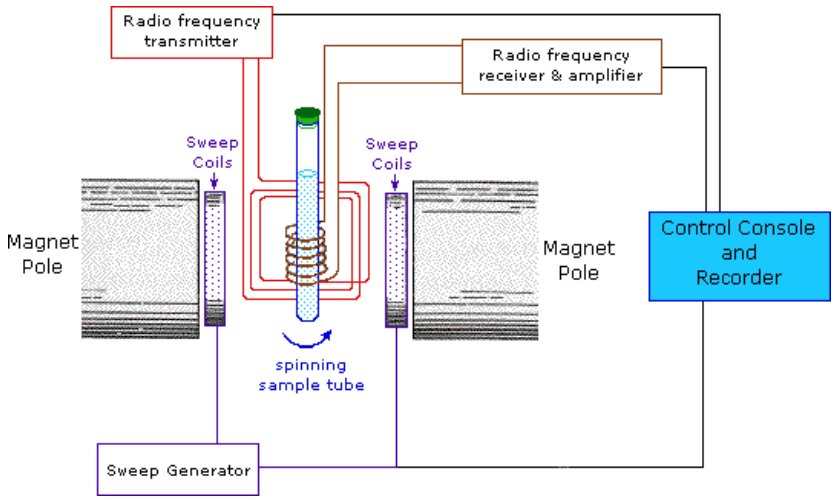

Figure. 3.2: Instrumentation of NMR.

LC-NMR

In a typical LC-NMR system, the LC unit consists of an auto sampler, the pump, column, and non-NMR detector (like DAD, UV, refractive index, or Electron Capture detector). From the

detector, the flow of eluent is directed to the interface of LC-NMR. During a LC-NMR operation, reversed phase columns are used and they utilize a binary or tertiary solvent mixture with isocratic or gradient elution method. The proton in the solvent used pose hindrance in obtaining an adequate NMR spectrum. The receiver of the NMR spectrometer is unable to difference between the strong solvent signals and the weak substance signals simultaneously. This drawback is overcome by solvent signal suppression that is attained by soft-pulse multiple irradiation.

4. Instumentation of LC-Nmr

4.1: Includes

- Solvent
- Pump
- Injector
- Column
- Detector
- Recorder

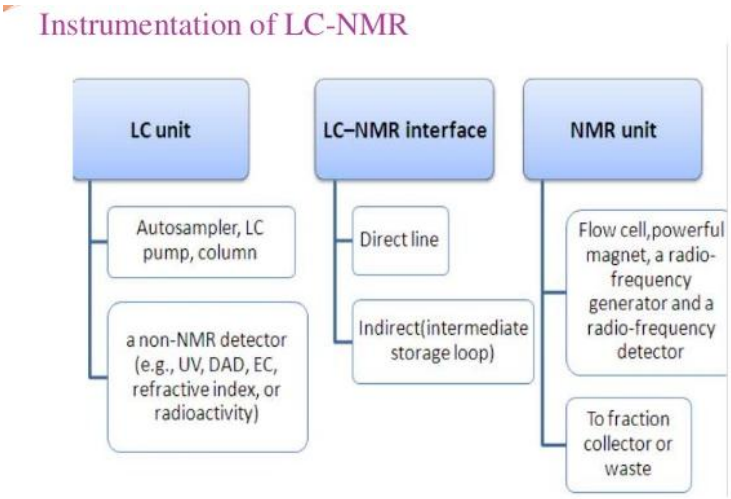

Figure. 4.2.: Instrumentation of LC-NMR.

- **Solvent:** A glass which contain of mobile phase (SAMPLE) usually contain both the mixture of polar liquid and non polar liquid components with respective concentration are varied depending on the composition of sample.**(Fig:4.1).**
- **Pump**: A pump aspirates the sample from the solvent reservoir and forces it through the systems column and detector. Depending on number of factors included column dimensions,

particle size of stationary phase, flow rate and composition of the mobile phase, operating pressure of up to 42000kPa(about 6000 psi) can be generated.

- **Sample Injector**: The injector can be single or automated injections. An injector of HPLC provide injection of the liquid sample in the range of 0.1-100ml of volume with high reproducibility and under high pressure up to 4000psi.
- **Column**: These are commonly filled with a stationary phase with particle size of 3-10um. Column usually made up of polished stainless steel with diameter of <2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column constant kept constant during analysis.
- **Detector**: The HPLC detector which are commonly used are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detector. It is located at the end of the column detect the analytes the elute from the chromatographic column.
- **Recorder**: Also called as data collection devices. Recorder-record the signals from the detector, by chat recorder or electronic integrators. The computer integrate the response of the detector to each component and places it into a chromatograph that is easy to read and interpet.

4.2 LC-NMR Interfaces

- **Direct Coupling:** which includes direct flow of LC effluent in to NMR flow cell and continuous recording of spectra.**(Fig:4.2a).**
- **Post-Column Splitter**
- **Value-Switching** INTERFACE i.e., BNMI (bruker NMR-mass spectrometry interface) **(Fig:4.2b).**
- **Indirect Coupling**: Intermediate storage loop which transfer outlet of LC to NMR flow cell at specified time interval. This can be done by Solid phase Extraction tmethod.

Figure. 4.2b. Bruker NMR-mass spectrometry.

4.3: NMR Units

NMR instrumentation involves the following units. A magnet to separate the nuclear spin energy state. Two RF channels, one for the field/frequency stabilization and one to supply RF irradiating energy. A sample probe containing coils for coupling the sample with the RF field; it consist of sample holder, RF oscillator, sweep generator and RF receiver. A detector to process the NMR signals. A recorder to display the spectrum.

4.4: Magnets

It is used to supply the principle part of the field Ho, which determines the Larmor or processional frequency of any nucleus. The stronger the magnetic field, greater the line separation of chemically shifted nuclei on the frequency scale. The relative populations of the lower energy spin level increase in the sensitivity of the NMR experiment.

- **Features**

It should give homogeneous magnetic field i.e., the strength and direction of the magnetic field should be constant over longer periods. The strength of the field should be very high at least 20,000gaus.

- **Types of Magnets**
- PERMANENT MAGNETS
- ELECTRO MAGNETS and
- SUPER CONDUCTING MAGNETS.

- **Super Conducting Magnets (SCM)**

A big stainless steel Dewar Persistent superconducting magnets are used to generate the Bo field; at low temperatures (<6K, typically) the resistance goes to Zero in the wire of super conducting. To maintain the wire in its superconducting state the coil is immersed in a bath of liquid helium(He)(4k, expensive). The “heat shield” kept at 77k by contact with a bath of liquid nitrogen(cheap) to reduce the amount of liquid helium boils off; Vacuum flask so as to further reduces the heat flow.

- **Advantages**
- strongest magnet.
- Stable & homogeneous magnetic field B0.
- Low running cost.

4.5: Magnetic Coils

It is not easy or convenient to vary the magnetic field of large stable magnets, however this problem can be overcome by superimposing a small magnetic field on the main field. Using a pair of Helmholtz coils (a Helmholtz coil is a device for producing a region of nearly uniform magnetic field, named after the German von Helmholtz) on the pole faces of the permanent does this. These coils induces a magnetic field that can be varied by varying the current flowing through them. The small magnetic field is producing in the same direction as the main field and is added to it. The sample is exposed to both fields, which appear one field to the nucleus.

4.6: Probe Unit

It is sensing element of the spectrophotometer system. It is inserted between the pole faces of the magnet in X-Y plane of the magnet air gap an adjusting probe holder. So the sample in NMR experiment experiences the combined effect of two magnetic field i.e., Ho and RF(EMR).[9] The usual NMR sample cell is generally made up of glass, which is strong and cheap. It consist of a 5 mm outer diameter and 7.5cm long glass tube containing 0.4mlof liquid. The sample tube in NMR is held vertically between the poles faces of the magnet. The probe contains a sample holder, sweep and detector coils, with the reference cell. The detector and receiver coil are oriented at 90 to each other. The sample probe rotates the sample tube at a 30-40 revolutions on the longitudinal axis. Each part of the sample tube experience the same time average the field.

4.7: Radio Frequency Generator

Using an RF oscillator creates the radio frequency radiation, required to induce transition in the nuclei of the sample from the ground state to excited states. The source is highly stable crystal controlled oscillator. It is mounted at the right angles to the path of the field of wound around the sample tube perpendicular to the magnetic field to get maximum interaction with the sample. The oscillator irradiates the sample with Rf radiation. Radio frequencies are generated by the electronic multiplication of natural frequency of a quartz crystal contained in a thermal stated block. To achieve the maximum interaction of the RF radiation with the sample, the coil of oscillator is wound around the sample container. The RFO coil of oscillator is installed perpendicular (90˚c) to the applied magnetic field and transmits radio waves of fixed frequency such as 60,100,200, or 300MHz to a small coil that energies the

sample in the probe. This is done so that the applied Rf field should not change the effective magnetic field in the process of irradiation.

4.8: Shim Coils: High resolution NMR requires line widths of <= 1.Magnetic field across the sample must be homogeneous so that the corresponding variation in the Larmor frequency is small. Surround the sample with a set of shim coils, each of which produces a tiny magnetic field with a particular spatial profile to cancelling out the small residual in homogeneities in the main magnetic field. Modern spectrometers might have up to 40 different shim coils labelled according to the field profiles they generates, such as x, y, $z,z^2,z^3,xy,xz,yz,x^2-y^2$ etc. Shimming, the process to optimize the shims, requires skill and experience because various shims will interact with each other.

The Transmitter Channel: Synthesizer: Rf source which produces a stable frequency which can be set precisely. RF amplifier: boost this small signal to a power of 100W or more to provide enough energy to excite the NMR active nuclei in the sample. Attenuator : altering the RF power level in units of decibels(dB).All under computer control.

5. Modes of LC-NMR

- **Continuous flow (on flow):** Eluent sampled in "real-time" as flowing through NMR detection coil.
- **Stopped flow:** pump is stopped at desired location and data acquired.
- **Time slices**: peaks, or "time-slices" of interest are analyzed**.**
- **Peak packing:** peaks of interest are "parked" in off-line sample loops.

Peak trapping: solid phase extraction cartridge are used to "re-concentrate" samples.

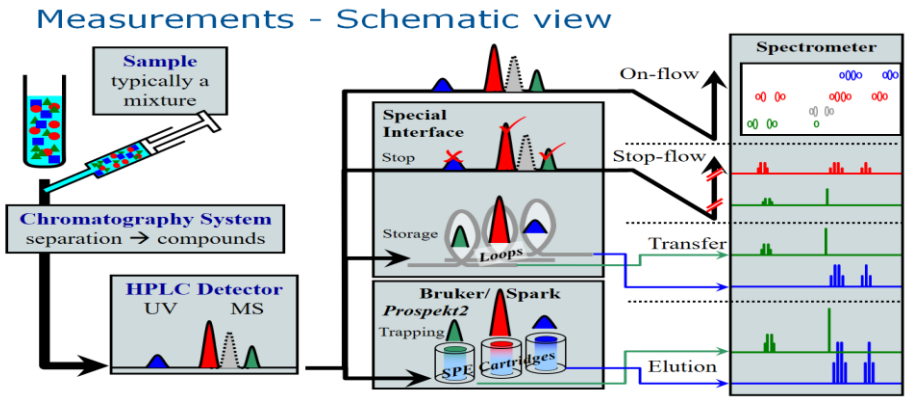

Figure. 5.2: Measurements Schematic view.

ON-Flow Mode[11]

- The outlet of the LC-detector directly to the NMR probe. While the peaks**(Fig 5.1)** are eluting, NMR spectra are continuously acquired. The chromatographic system is used to move the samples/peaks through the NMR cell.

Equipment: Any HPLC system, which delivers a stable pulse free flow.

- LC-NMR probes.
- LC-NMR interface not acquired.
- With any of the LC-NMR interface this working mode is also possible, however they are not required**.**

Stopped Flow Method

The outlet of the LC-detector is connected directly NMR probe.**(Fig5.2)** A LC-detector (normally UV) is used to detect peaks eluting from the column. When a peak is detected, the flow continues until the peak arrives in the NMR cell. At this time, the chromatography (pump, data acquisition, gradient) stops and, the NMR experiments are performed. Once the NMR experiments are completed, the chromatography resumes until the next peak is found. This process can be repeated several times within one chromatogram.

Equipment: HPLC system, controlling system.

Time Slice Method

It includes to stop the flow at short interval over the chromatography peak to time slicedifferent part of chromatography run. It is useful if there is poor chromatography separation or if compound under study have poor or no UV chromophore or if the extract chromatography retention time is unknown. The data from such a time slice experiment referred as a total NMR chromatogram (tNMRc).

- **Peak Parking Method**

The outlet of the LC- detector is connected to the sample loops of the BPSU-36 or BPSU-12. A LC- detector (normally UV) is used to detect peaks eluting from the column. A detected peaks is moved into one of the sample loops without interrupting the chromatography. When the chromatography is completed, the HPLC pump is used to transfer the peaks from the loops into the NMR probe.

Equipment: any HPLC system Pump under control for transfer.

LC-NMR probe BPSU-12(bruker peak sampling unit-12) Controlling station.

Peak Trapping Method

The outlet of the LC-detector(normally UV) is used to detect peaks to the SPE unit. A LC-detected peak is moved trapped on a SPE cartridge without interrupting the chromatography When the chromatography is completed, the chromatography solvents are removed and the peak is transfer with fully deuterated solvents into the peak is transfer with fully deuterated solvents into the NMR probe.

Equipment: any HPLC system.

Pump under control for transfer

LC-NMR probe

SPE system Controlling station.

6. Technology to Improve Sensitivity of LC- Nmr Method

6.1 LC Method

- On line SPE method
- Online column trapping method
- Use of semi-micro method

6.2 NMR Method

High strength magnetic field and high sensitivity probe.

6.3 Solvent Suppression Column

- Presaturation
- Soft pulse multiple irradiation.
- Wet method.

6.1a ON-Line Spe Method

This method is widely used in LC-NMR as a method for concentrating trace components.[12] The Spark Holland SPE system can be controlled through the same interface as LC-NMR and SPE cartridge absorbs the desired peak. After the sample is dried with nitrogen gas, it is eluted by a small amount of solvent of several tens of micro liters, and a highly concentrated target component can be introduced to the flow-cell. It is possible to fractionate multiple components continuously in different cartridges, and it is compatible with simultaneous analysis.

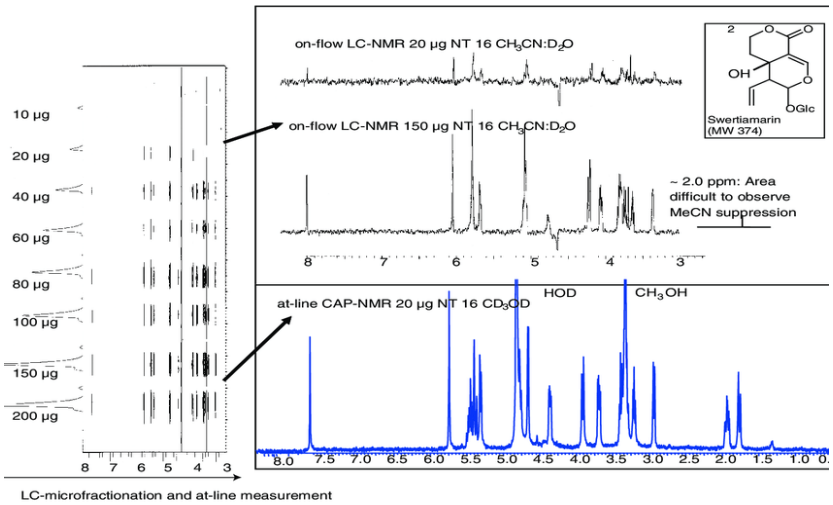

Figure. 5.1. Lc-microfraction and at-line measurement.

6.1b Online Column Trapping Method

It is used as a peak concentration for LC-NMR. After separation using a conventional column, Concentration is 1st done in trap column and the sample is separated again using a semi-micro column then introduced to NMR concentration. This technique is highly effective and two dimensional NMR measurements. Which are usually attended with much difficulty caused by low sensitivity can be achieved.

Conventional column and semi-micro columns with identical fillers can be obtained easily from various column manufactures and since the separation pattern does not differ from the conventional columns, this method is easy to used from the stand point of not needing additional investigations into conditions other than matching the flow rate. The method of components being separated by preparative columns and concentrated in trap columns, then being introduced to LC-NMR after separation using a conventional column is also known as online column trapping.

6.1c Semi-Micro Column Method

SPE and semi-micro column reduce the amount of usage of expensive deuterated solvents, and interference due to signals originating in non-deuterated solvents is held to a minimum. Improvement of solvent suppression efficiently.

6.2: Highly Magnetic Field Magnets and Highly Sensitivity Probes

It have made a large contribution to the equipment accept in the achievement of high performance LC-NMR. NMR detection sensitivity is proportional to the magnetic field

strength to the 3/2 power and the stronger the external magnetic field is the higher the sensitivity.[13] The improvement of magnetic field strength is also a central problem for LC-NMR. The magnetic field strength has reached 800 MHz[14] or more, and compared with 60MHz equipment in 1978 and this has made for a 50 fold improvement in sensitivity. High sensitivity probe known has a cryogenic probe[15] the reduces the heat noise arising during NMR signal detection by cooling the coil using super conductor materials. The elapsed time required for acquiring the ^1H NMR spectrum of a trace component with a content of 0.1% with the current 500 MHz equipment is 20hrs or longer, while with 800MHz equipment it is only around 30minutes.

Solvent Suppression Column[6]

Pre-saturation: The most widespread solvent suppression in use called as pre-saturation. A highly selective low power pulse irradiates the desired solvent signal for 0.5 -2s, where no irradiation occur. This method used as stopped flow mode.

- **Soft pulse multiple irradiation**

Pre-saturation is performed with the use of shaped pulse which has broader excitation profile. Advantage: Easy to apply and easy to implement with most of the NMR experiment, and multiple pre-saturation is possible, and that it is very effective.

Disadvantage: transfer of saturation can occur to slowly exchanging protons that would be detectable without saturation. That spin with resonance close to solvent frequency will also be saturated and 2D cross peaks will be occur.

- **Wet method[16]**

This method combines selective excitation pulse and pulsed field gradient which can suppress multiple solvent peaks, If solvent elimination method that is currently widely used in LC-NMR. When solvent signals does not directly overlap the signals of the target compound, then the signals from trace components is not detected because of the dynamic range when the solvent signals is not eliminated.

- **Examples of solvent suppression method**

Tetrachloride, Tetra-chloro ethylene, Freon where it does not contain hydrogen.

Advantages: HPLC resolves complexity of a mixture where NMR resolves virtually any structure question in which separation between two phases. LC-NMR is the ultimate

instrument where it determines the LC peak levels where data can be taken without complete separation of mixture. Where the sample can be store for another method for analysis.

Disadvantage

- High cost effect.
- Difficult in solvent selection.
- Long experiment time; partial use of 2H solvent.
- Operator training requirement.

7. Applications

- Extract crude drug from plant derived products.[17]
- A multi-technique approach using LC-NMR for the isolation and characterization of low-level unknown impurities in GW876008, a novel corticotrophin release factor 1 antagonist.[18]
- Characterization of all impurities present in the drug.
- Detection or isolating using other techniques.
- Studying of drug metabolism-analysis of bio-fluids such as plasma, HIV-1 reverse transcriptase inhibitor BW935U83.[19]
- Studying polymers- the high resolution of LC-NMR for analysis of the microstructure in synthesis polymer and biopolymer.
- Application of evolving factor analysis to on-flow LC-NMR data using spectral windows.[20]
- Application of LC-MS and LC-NMR to the identification of degradation products of a protease inhibitor in dosage formulations.[21]
- Characterization of triacetone triperoxide (TATP) conformers using LC-NMR.[22]

8. CONCLUSION

Hyphenated techniques is the most powerful strategy that an analyst can use to study complex mixtures of natural products. The different possible modes of operation and the various combination of detectors and sample manipulation techniques available that restrict on the applicability of LC-NMR can be reduced to a minimum The type of sample and the goals of the analysis determine the optimal choice of instrumentation and measurement mode. Identification of substances in simple mixtures, the on-flow techniques are more efficient. The sample amount is limiting LC-NMR holds much promise for advance in the fields of plant metabolism and natural- products analysis. Detailed LC-NMR investigations of medicinal plants could contribute to new leads to drug development.

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